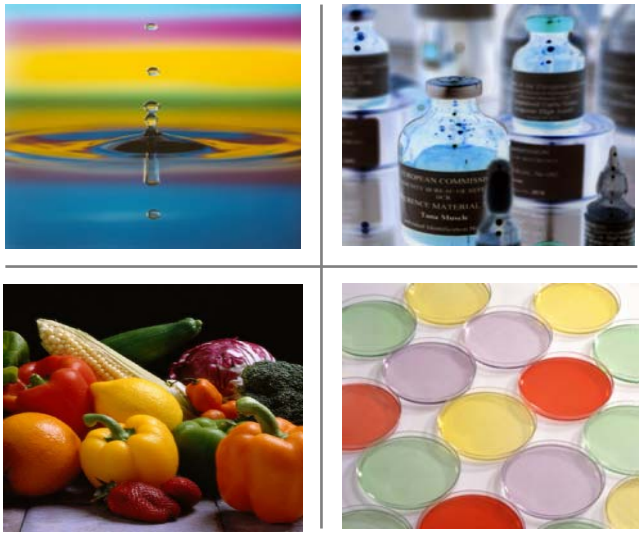
EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Institute for Reference Materials and Measurements

Guidance Document on Measurement Uncertainty for GMO Testing Laboratories

S. Trapmann, M. Burns, H. Broll, R. Macarthur, R. Wood, J. Zel



Report EUR 22756 EN

The mission of IRMM is to promote a common and reliable European measurement system in support of EU policies.

European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
S. Trapmann
European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements
Retieseweg 111
B-2440 Geel • Belgium

E-mail: Stefanie.trapmann@ec.europa.eu

Tel.: +32 (0)14 571 714
Fax: +32 (0)14 571 548

http://www.irmm.jrc.be
http://www.jrc.ec.europa.eu

Legal Notice
Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use which might be made of the following information.

A great deal of additional information on the European Union is available on the Internet.
It can be accessed through the Europa server
http://europa.eu.int

EUR Report 22756 EN
ISBN 978-92-79-05566-9
ISSN 1018-5593
Luxembourg: Office for Official Publications of the European Communities

© European Communities, 2007

Reproduction is authorised provided the source is acknowledged

Printed in Belgium

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Institute for Reference Materials and Measurements

Guidance Document on Measurement Uncertainty for GMO Testing Laboratories

S. Trapmann, M. Burns, H. Broll, R. Macarthur, R. Wood, J. Zel

This guidance document has been elaborated on request of the European Network of GMO Laboratories (ENGL) to further harmonise the estimation of measurement uncertainty of real-time PCR

Summary

This technical report outlines the technical issues related to the estimation of measurement uncertainty (MU) involved in the GMO sector. In particular it gives guidance to GMO testing laboratories how to estimate the analytical variability of quantitative analytical results obtained by real-time PCR. This guidance document has been written on request of the European Network of GMO Laboratories (ENGL) as a follow-up of a workshop on Measurement Uncertainty in the GMO sector organised by the Institute for Reference Materials and Measurements (IRMM), Geel, Belgium and held on 05.07.2005.

It is recognised that in order to be able to judge if an analytical results exceeds a threshold; the MU must be estimated and reported together with the measurement result. Enforcement Authorities shall therefore estimate the MU associated with an analytical result and use it to decide whether an analytical result falls within the specification of food and feed control. The value obtained by subtracting the expanded uncertainty from the reported concentration is used to assess compliance. Only if this value is greater than the legal threshold, it is sure 'beyond reasonable doubt' that the sample concentration of the analyte is beyond what is permissible.

Two selected approaches for the estimation of MU are presented in detail; references to alternative approaches are given. The first approach presented in detail is using data from collaborative trial in combination with in-house quality control data for the estimation of MU. Prerequisites for the use of such collaborative trial data are outlined. In case no suitable collaborative trial data are available, an alternative approach for the estimation of MU, using data obtained on within-laboratory samples, is presented.

Table of Content

Glossary

AOAC	Association of Official Analytical Chemists
c	sample concentration
CRL	Community Reference Laboratory
CRM	Certified Reference Material
CTAB	cetyltrimethylammonium bromide
Ct-value	number of PCR cycles to pass a set threshold
DG SANCO	Directorate-General Health and Consumer Protection
DNA	deoxyribonucleic acid
ENGL	European Network of GMO laboratories
ERM®	European Reference Material®
EU	European Union
EURACHEM	Network of analytical chemistry organisations in Europe
GM	genetically modified
GMO	genetically modified organism
GUM	Guide to the Expression of Uncertainty in Measurement
IQC	internal quality control
IRMM	Institute for Reference Materials and Measurements
ISO	International Standards Organisation
IEC	International Electrotechnical Commission
IUPAC	International Union of Pure and Applied Chemistry
LC	critical level
LOD	limit of detection
LOQ	limit of quantification
m/m	mass fraction
MU	measurement uncertainty
n	number of independent measurements
NIB	National Institute of Biology
NMKL	Nordic Committee on Food Analysis
PCR	polymerase chain reaction
RSU	relative standard uncertainty
RRS	Roundup Ready® soya
TVP	texturised vegetable protein
U	expanded uncertainty
u	standard uncertainty

1 Introduction

It has been recognised that there are a number of actions that may be taken by those responsible for the enforcement of EU legislation which directly affect decisions as to whether a sample, or a lot from which a sample is taken, is in compliance with EU legislation.

It must be appreciated that the enforcement of any requirement in EU legislation which is based on measurement results depends on the analytical methods and their measurement uncertainties (MUs). Without common and uniform criteria for evaluation and interpretation of the MU, different Member States will make different judgements as to whether any particular lot is in compliance with its EU specification.

After a workshop on MU in the GMO sector organised and held on 05.07.2005 at the Institute for Reference Materials and Measurements (IRMM) in Geel, Belgium, a task force of the European Network of GMO Laboratories (ENGL) has been formed to outline the related scientific and technical issues. This resulting technical report provides recommendations and gives guidance to the Enforcement Authorities in Member States on procedures to be adopted that reduce the possibility of Member States taking differing views as to whether a particular sample is in compliance with any particular EU specification.

This report focuses on analytical issues only. In particular it considers the treatment of analytical uncertainty (normally known as the MU) in the interpretation of a specification. It is mainly concerned with quantitative analytical results. This report is written in a form such that the complex issues involved can be readily appreciated by all. In particular, it:

- sets out the issues;
- gives recommendations for consideration by the Enforcement Authorities of the Member States; and
- gives a series of technical Annexes to aid practitioners in estimating their MUs.

This report further develops the general DG SANCO report 'On the relationship between analytical results, measurement uncertainty, recovery factors and the provisions of EU food and feed legislation, with particular reference to community legislation concerning contaminants in food and undesirable substances in feed' [1] for application in the GMO sector.

It should be noted that the MU estimation as such is independent from the unit of measurement. Independent if mass fractions (m/m) or copy number ratios are used, the estimation is carried out in the same way. Attention needs to be paid, that the unit of measurement is not changed during its uncertainty estimation. However, the user should pay extra attention when using percent as measurements unit as these absolute values can easily be mixed-up with relative values given as well in percent.

[1] DG Health and Consumer Protection: Report to the Standing Committee on the Food Chain and Animal Health on the relationship between analytical results, the measurement uncertainty, recovery factors and the provisions in EU food and feed legislation with particular focus on the community legislation, http://ec.europa.eu/food/food/chemicalsafety/contaminants/report-sampling_analysis_2004_en.pdf

1.1 Purpose

The requirements and recommendations made in this document are aimed at providing help to the practitioner dealing with samples under Regulation (EC) No 882/2004 'on official controls performed to ensure the verification of compliance with feed and food law' [2] and specifically under Regulation (EC) No 1829/2003 on 'genetically modified food and feed' [3] and Regulation (EC) No 1830/2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms' [4]. In addition these recommendations should be applied for seed. Only detection procedures which are intended for an event-specific quantification (e.g. by real-time PCR approach) of material derived from a GMO are covered.

1.2 Procedures for the estimation of measurement uncertainty

MU is generally thought of as applying to quantitative measurements. Thus, in the GMO sector it will apply to the quantitative estimation of the 'concentration' (however expressed) of an authorised GMO. The concept will also apply qualitatively in the case of both authorised and non-authorised GMOs (i.e. confirmation of presence/absence). This latter aspect is increasingly being recognised as being of importance, but work on 'Qualitative Measurement Uncertainty' is only just commencing at the international level [5].

MU, which should take account of all effects on a measurement process, is the most important single parameter that describes the quality of measurement. The MU is linked to the individual measurement performed but not to a real-time PCR method as such. Therefore each laboratory has to evaluate the specific MU for a measurement results obtained under defined conditions. The uncertainty arises both from sampling and analysis, unless it could be proven that the sampling carried out is representative and that sampling uncertainty can therefore be neglected. It should be noted that generally a large amount of MU can arise from the upstream sampling stage, this is however not the rationale of this document and this guidance document addresses only the MU arising downstream the sampling stage.
Significant progress has been made in devising procedures to estimate the uncertainty that originates in the analytical portion of the measurement, and guidance on these procedures is available. It has, however, become increasingly apparent that sampling is often an important contribution to uncertainty and requires equally careful management and control. Thus, the uncertainty arising from the sampling process should be evaluated. While existing guidance identifies sampling as a possible contribution to the uncertainty in a result, procedures for estimating the resulting uncertainty are not well developed and further, specific, guidance is required. At this time this guidance document will only consider analytical MU, but will be revised as further information, most notably that from the EURACHEM Working Group

[2] Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules, Official Journal of the European Union, L 165
[3] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed, Official Journal of the European Union, L 268/1
[4] Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC, Official Journal of the European Union, L 268/24
[5] IUPAC Project 2005-024-2-600 (Guidelines for validation (interlaboratory) of qualitative methods)

dealing with sampling uncertainty, becomes available. The issue has also been discussed in the Codex Committee on Methods of Analysis and Sampling [6].
It is important to recognise that there is always a MU associated with an analytical result reported by a control laboratory, whether or not the MU is reported. It should be noted that control laboratories testing for compliance with regulations (EC) No 1829/2003 and 1830/2003 must report MU uncertainty together with the measurement result.

The first recognised approach to MU, the 'Guide to Expression of Uncertainty in Measurement' (GUM) [7] lays down general rules for the expression and estimation of MU. GUM introduces the idea of uncertainty and distinguishes it from errors. It furthermore describes the steps involved in the estimation of uncertainty. GUM places emphasis on the component-by-component approach, in which the method is dissected and incremental calculations of uncertainty are made and eventually added up to provide a combined uncertainty.
The evaluation of the measurement uncertainty for a method requires the analyst to look closely at all the possible sources of uncertainty in the method concerned; this approach is called 'bottom-up approach'. As a visualisation tool cause and effect diagrams also named 'fishbone diagrams' are used and practical studies are carried out to help identifying the major source of uncertainty associated with the measurement. Figure 1 is giving examples of possible sources of MU for DNA quantification by real-time PCR. By concentrating on the major sources of uncertainty a good estimate of the uncertainty as a whole can be achieved, for further details the reader is referred to other documents exploiting this approach [8].
Once the measurement uncertainty has been estimated for a certain method in a particular laboratory, this estimate can be applied to subsequent results, provided that they are carried out in the same laboratory under the same conditions and that quality control data justify the correctness of this approach. In practice collaborative trail data are used to verify that the 'bottom-up approach' chosen covers sufficiently all potential variations, if this is not the case major sources of uncertainty were incorrectly identified.

[6] CCMAS (April 2006): Uncertainty of Sampling, CX/MAS 06/27/10
[7] ISO (1995): Guide to Expression of Uncertainty in Measurement, ISBN-9267-101889
[8] EURACHEM / CITAC (2000): Quantifying Uncertainty in Analytical Measurement, second edition, EURACHEM Secretariat, BAM, Berlin (http://www.eurachem.ul.pt)

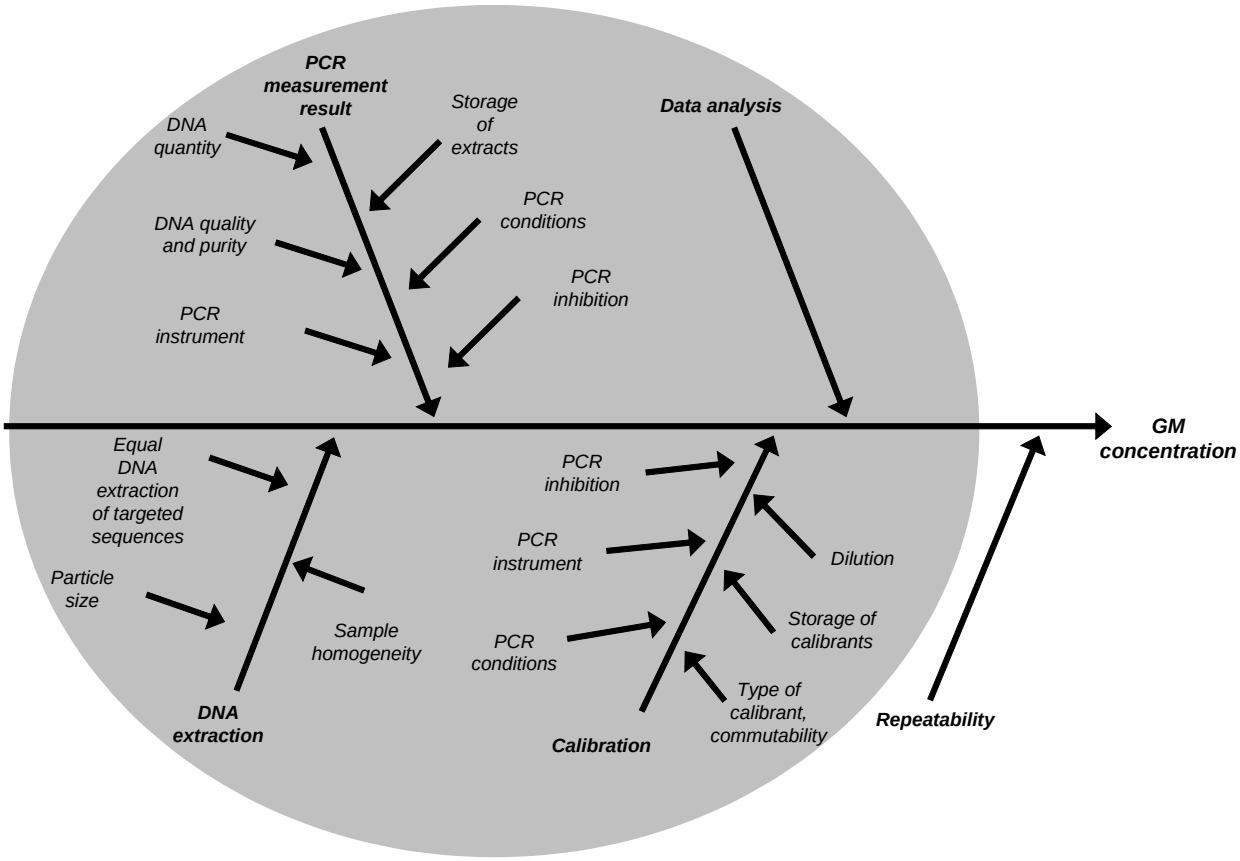


Figure 1: Cause and effect diagram ('fishbone diagram') visualising examples for possible MU contributions for DNA quantification by real-time PCR. Contributions to the reproducibility are shaded grey.

It is desirable that information available as a result of accreditation requirements is used by the laboratories when estimating MU in order to avoid duplicate work being carried out. Additionally there has been some criticism of the practicability of the 'bottom-up approach' for the estimation of MU and two alternative ways are presented in this document.
In the GMO food and feed sectors, where there is a high emphasis being placed on the use of methods of analysis validated through collaborative trials, information obtained from such trials can be used in many situations to estimate MU. However, it is important to note that data from collaborative trials can only be used if the findings are confirmed by internal quality control procedures.

It is recommended that GMO laboratories use information derived from the following procedures to aid their estimation of the uncertainty of measurement results:

- The ISO Guide to expression of uncertainty in measurement [7]
- The EURACHEM Guide to quantifying uncertainty in analytical measurement [8]:
    A. component-by-component approach
    B. use of collaborative trial and/or internal quality control data

These documents recommend procedures based on a component-by-component approach, method validation data, internal quality control data and proficiency test data.
In many cases the overall uncertainty may be evaluated by an inter-laboratory (collaborative) trial by a number of laboratories and a number of matrices:

- IUPAC/ISO/AOAC International protocol for the design, conduct and interpretation of method performance studies [9]
- ISO 21748 guidance document for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation [10]
- ISO/IEC 17025 outlining the internal quality control approach [11]
- Nordic Committee on Food Analysis (NMKL) suggesting the use of experimental data generated within the individual laboratory [12]
- Nordtest report outlining the use of data obtained on routine samples for the estimation of MU [13]

The above, and others, notably:

- The concept set by Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results [14]
- An AOAC international approach [15] is outlined in the DG SANCO MU report [1].

It is recognised that further procedures for the estimation of MU may exist and are being developed, and that, in this evolving situation, further recommendations will be made as to acceptable procedures. As an example, it is anticipated that procedures based on results obtained from participation in proficiency testing schemes will be developed.

[9] Horwitz W (1995): Protocol for the Design, Conduct and Interpretation of Method Performance Studies, Pure Appl. Chem., 67, 331-343
[10] ISO/TS 21748 (2004): Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation
[11] ISO/IEC 17025 (2005): General requirements for the competence of testing and calibration laboratories
[12] NMKL (1997): Estimation and expression of measurement analysis in chemical analysis, procedure No5
[13] Magnusson B, Näykki T., Hovind H, Krysell M (2004): Handbook for Calculation of Measurement Uncertainty in Environmental Laboratories, TR 537 of 2004-02
[14] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Official Journal of the European Union L221 (Text with EEA relevance) (notified under document number C (2002) 3044)
[15] Horwitz W (2003): The Certainty of Uncertainty Journal of AOAC INTERNATIONAL, 86, 109-111

1.3 State of the art of collaborative trials in GM quantification

Currently, more than 23 collaboratively tested quantitative real-time PCR-based methods, with at least 6 participants per trial, have been published. The majority of collaboratively tested methods were conducted with sample and calibration material such as ground seed as it is available from IRMM. Material from the same source was used as unknown sample and as material for the establishment of the calibration curve. Hence, any matrix effect which might have an influence on the MU will not be identified by this approach. Some trials were conducted using DNA that was simply provided by the organising institute and do not cover the DNA extraction step. Several trials use a common source of primer, probes and polymerase, which leads as well to a lower value for the reproducibility. In case only one type of PCR instrument is used the reproducibility investigated is only valid for this type of instrument. Additionally it should be noted that collaborative trials are never carried out under routine conditions.

The potential bias associated with results may not reflect real measurements, where the calibration matrix may not exactly match the analytical sample matrix. In order to estimate the size of such effects a reconciliation procedure can be used. For an example see Annex II.

In general, a list of collaborative trials including results can be found in ISO standard 21570 [16]. Additionally method validations carried out by the Community Reference Laboratory (CRL) for GMO food and feed can be found on the CRL homepage [17]. Codex Alimentarius Commission is currently developing a document called 'Consideration of the methods for the detection and identification of foods derived from biotechnology', which will list as well methods validated through collaborative trial.

[16] ISO 21570 (2005): Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Quantitative nucleic acid based methods
[17] Homepage of the Community Reference Laboratory for GMO food and feed: http://gmo-crl.jrc.it

2 The relationship between the analytical result and measurement uncertainty, with particular reference to regulation (EC) No 1829/2003 and (EC) No 1830/2003

2.1 General description of the estimation and use of measurement uncertainty

The general process of estimating and using MU entails:

- the evaluation of all possible sources of uncertainty in the method concerned and identification and combination of the major sources of uncertainty ('bottom-up approach'); or alternatively
- the use of collaborative trial and/or single-laboratory validation data and/or in-house quality control data to estimate relation between sample concentration (c) and standard uncertainty (u); and
- the calculation of the Critical Level (LC), Limit of Detection (LOD) and Limit of Quantification (LOQ) from u

The estimation of MU is achieved by the estimation of two values u_0, the standard uncertainty associated with results of the measurement of low concentrations of GM, and *RSU* the relative standard uncertainty associated with the results of the measurement of high concentrations of GM (Section 2.4). The value for u_0 can be estimated by extrapolation, provided that measurement results for samples close to the LOQ are available. In the other case the analysis of diluted samples should be considered. *RSU* can be estimated using collaborative trial data, in-house quality assurance data or single laboratory validation data. Examples based on collaborative trial and in-house quality assurance data are given below.

The estimates of the standard uncertainty can be based on collaborative trial data, provided the laboratory can confirm, via internal quality control (IQC) data, that it is able to achieve the same performance of the laboratories that took part in the trial. If the estimate of the standard uncertainty derived from collaborative trial does not cover the complete reproducibility due to the set-up of the trial, a reconciliation procedure should be used to estimate the contribution of effects not investigated in the trial (Annex II). Each significant source of uncertainty not covered by the collaborative trial data should be evaluated in the form of a standard uncertainty and combined with the reproducibility standard deviation [8]. During the 'reconciliation' stage, it is necessary to identify any sources of uncertainty that are not covered by the collaborative trial data. The sources which may need particular consideration are [8]:

• Sampling: Collaborative trials rarely include a sampling step. If the method used in-house involves sub-sampling, or the measurand is estimating a bulk property from a small sample, then the effects of sampling should be investigated and their effects included.
• Pre-treatment: In most trials, samples are homogenised, and may additionally be stabilised, before distribution. It may be necessary to investigate and add the effects of the particular pre-treatment procedures applied in-house.
• Method bias: Method bias is often examined prior to or during interlaboratory study, where possible by comparison with reference methods or reference materials. Where the bias itself, the uncertainty in the reference values used, and the precision associated with the bias check, are all small compared to the repeatability standard deviation (s_R), no additional

allowance need be made for bias uncertainty. Otherwise, it will be necessary to make additional allowances.

- Variation in conditions: Laboratories participating in a trial may tend towards the means of allowed ranges of experimental conditions, resulting in an underestimate of the range of results possible within the method definition. Where such effects have been investigated and shown to be insignificant across their full permitted range, however, no further allowance is required.
- Changes in sample matrix: The uncertainty arising from matrix compositions or levels of interferents outside the range covered by the trial will need to be considered.

In cases were no collaborative trial data are available, the MU can be estimated from analytical results measured within the laboratory concerned. Preference should, however, be given to data obtained through collaborative trials.

2.2 Calculation of measurement uncertainty from collaborative trial data and confirmed by internal quality control data

A collaborative trial is usually carried out once as a part of method validation, or at relatively infrequent intervals during the working lifetime of a method; it tells the analyst what performance they can expect for the method applied. However, attention needs to be paid that the MU estimated from a method validation carried out by collaborative trial covers all measurement steps contributing to the MU (Figure 2).

In order to use MU estimates from collaborative trials properly, it is important that the same replication (e.g. two PCRs of one DNA extract), more or less the same Ct values and design of the calibration curve are used to produce a single measurement result in the laboratory.

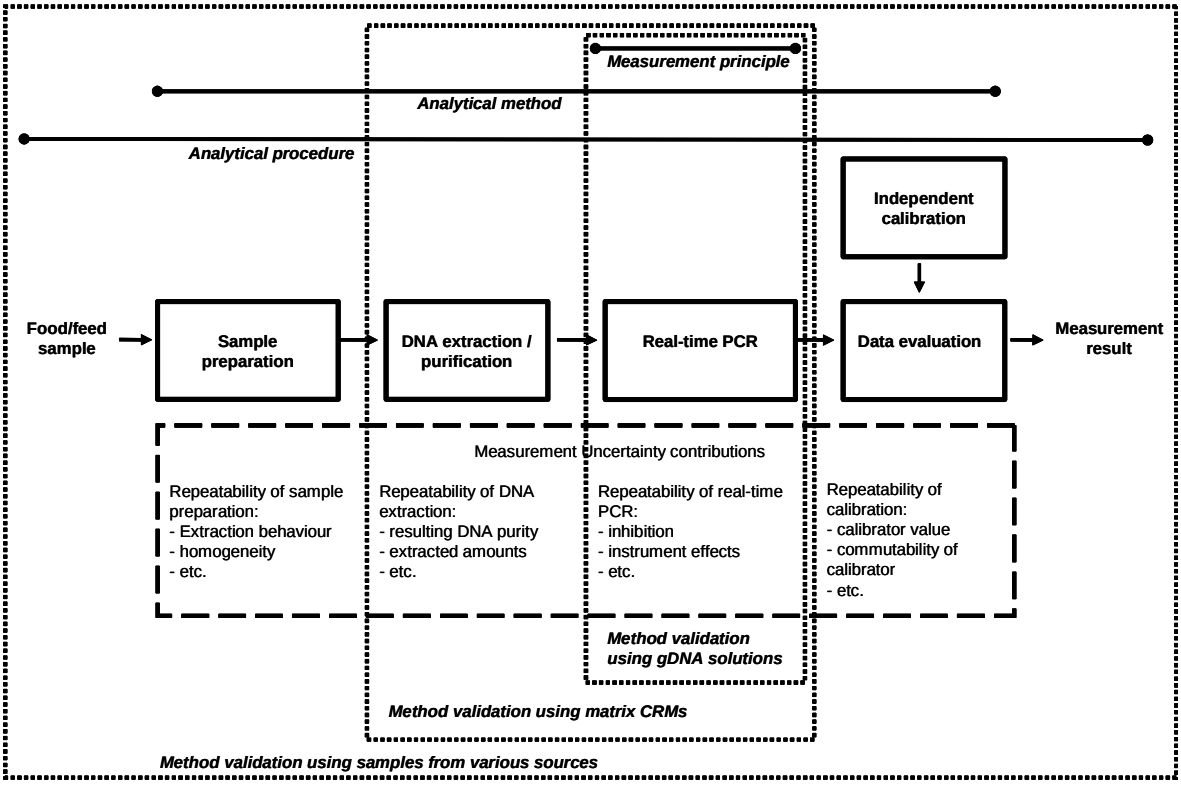


Figure 2: Contributions to the estimation of the MU within one laboratory

The purpose of IQC is to ensure that the performance of the analytical system remains effectively unchanged during its application. IQC is required for accreditation according to ISO/IEC 17025.

In general, s_R determined in a collaborative trial can be used as an estimate of MU if the following conditions are met:

- the collaborative trial studied the whole method using sample types similar to those being measured in the laboratory;
- repeatability in the laboratory is similar to the repeatability reported in the collaborative trial;
- within-laboratory reproducibility is not larger than the reproducibility reported in the trial;
- results from the analysis of Certified Reference Materials (CRMs) in the laboratory do not display significant bias, provided the relevant CRM properties correspond fully to those of the test sample;
- a true value has been assigned to the test material independently of the test results.

If these conditions are not met then estimates of the additional uncertainty components must be combined with the collaborative trial s_R to provide an estimate of MU.

Within this sector, collaborative trials are often undertaken using samples containing a range of concentrations of GM material, including the concentrations close to LOQ. The uncertainties u_0 and *RSU* can be estimated by plotting s_R of collaborative trials against the assigned value (or mean) of the concentration of GM and calculating the linear regression. The intercept of a linear regression (or s_R associated with the lowest concentration) can be used as an estimate of u_0 and the slope of the linear regression can be used as an estimate of *RSU*. If the intercept is negative or the lowest concentration is close to the LOQ, s_R associated with the lowest concentration can be used as an estimate of u_0.

CRMs are well suited for the estimation of bias and its control in IQC. Measurement variation is best estimated and controlled by the repeated independent analysis of samples. If a CRM is employed as an 'unknown sample' for IQC, material from a different source should be used for calibration.

2.3 Calculation of measurement uncertainty from internal quality control data

MU can be estimated using the value of within-laboratory reproducibility; provided it could be proven that no bias exists. The general approach is to estimate the value of within-laboratory reproducibility (measurement variation) using the repeated independent analyses of a range of real samples, and to carry out a bias control using CRMs. The use of normal quality assurance procedures and measurements results obtained on routine samples should mean that suitable data are readily available [12, 13]. For new methods, where quality assurance data is not available, suitable analytical results can be obtained from a within-laboratory method validation [13]. The approach is presented in Section 2.3.1.

In order to ensure that the analytical result and its uncertainty cover the true concentration of GM in a sample, it is important to prove that no bias exists.

An example for the calculation of MU based on internal quality control data is given in Annex III.

2.3.1 Within-laboratory reproducibility

The within-laboratory reproducibility can be estimated using measurements results obtained on routine samples as outlined in the NMKL procedure No. 5 [12] or the Nordtest report [13]. The following approach is based on the Nordtest report [13], a calculation example can be found in Annex III.

It is important that the estimation of MU covers all steps of the analytical procedure. Hence, within-laboratory reproducibility should be determined by the repeated independent analyses of samples in analytical runs that represent the long-term variation of analytical components within the laboratory, e.g. different operators, stock solutions, new batches of critical reagents, recalibrations of equipment. Also, samples should represent the different matrices and concentrations to which the estimates of MU will be applied. In particular, samples with a GMO content close to legal or contractual thresholds against which results will be compared should be included (e.g. 0.9 % for approved events in unlabelled food products).

Uncertainty estimates should be updated by the addition of new results as they become available. Once a database is established then it may be advisable to remove results that are older than one year, from the estimation of measurement uncertainty, if new results are generated frequently. In regular intervals revalidation should be considered.

Repeated independent results produced by at least 15 samples should be used. Usually there is a limited resource that can be devoted to measurements that are used to estimate uncertainty. Therefore, in order to maximise the matrices and concentrations studied it is recommended that the smallest replication (i.e. two independent measurements (n = 2) per sample,) are applied to the largest number of samples possible. In the context of the Nordtest [13] 'duplicate measurements' mean two independent examples of the measurement as it is usually applied in the laboratory. For example, if it is the usual practice to report results as the mean of two analyses then the two independent measurements will consist of two results each given by the mean of two analyses.

The mean ($\bar{c}_i$) of two independent analytical results is calculated as:

$$\bar{c}_i = \frac{c_{i,1} + c_{i,2}}{2} \qquad \text{Equation 1}$$

$\bar{c}_i$ ..... mean of two analytical result

$c_{i,1}$ ..... result of first analysis of sample i

$c_{i,2}$ ..... result of second analysis of sample i

The absolute difference (d_i) between the first and the second analysis is calculated as:

$$d_i = \left| c_{i,1} - c_{i,2} \right| \qquad \text{Equation 2}$$

d_i ..... absolute difference between two analytical result

$c_{i,1}$ ..... result of first analysis of sample *i*

$c_{i,2}$ ..... result of second analysis of sample *i*

The relative difference between analyses (rad_i) is calculated as:

$$rad_i = \frac{d_i}{\bar{c}_i} 100 \qquad \text{Equation 3}$$

rad_i ..... relative difference

d_i ..... absolute difference between two analytical result

$\bar{c}_i$ ..... mean of two analytical result

Given a set of differences and relative differences calculated from the analysis of a number of samples the average difference ($\bar{d}$) and average relative differences ($\overline{rad}$) can be calculated.

The within-laboratory s_R is in the case of two independent measurements results ($n = 2$) given by:

$$s_R = \frac{\bar{d}}{d_2} = \frac{\bar{d}}{1.13} \qquad \text{Equation 4}$$

s_R ..... within-laboratory reproducibility standard deviation

d_2 ..... constant depending on the number or independent measurements (n) [13]:

Table 1: Factor d_2 depending on the number of independent measurement results carried out on one sample

n	d_2
2	1.128
3	1.693
4	2.059
5	2.326
6	2.534
7	2.704
8	2.847
9	2.970
10	3.078

Note: In case more than two independent measurement results are measured, d_i reflects the range between the lowest and the highest results measured.

The within-laboratory reproducibility relative standard deviation (RSD_R) is given by

$$RSD_R = \frac{\overline{rad}}{1.13} \qquad \text{Equation 5}$$

RSD_R .... relative within-laboratory reproducibility

$\overline{rad}$ ..... average relative differences

2.3.2 Method and laboratory bias control

After the measurement of a CRM the absolute difference between the mean measured value and the certified value can be calculated as:

$$\Delta_m = |c_m - c_{CRM}| \qquad \text{Equation 6}$$

Δ_m ..... absolute difference between mean measured value and certified value
c_m ..... mean measured value
c_{CRM} ..... certified value

The uncertainty of Δ_m is u_Δ, that is calculated from the uncertainty of the certified value and the uncertainty of the measurement result according to:

$$u_\Delta = \sqrt{u_m^2 + u_{CRM}^2} \qquad \text{Equation 7}$$

u_Δ ..... combined uncertainty of result and certified value (= uncertainty of Δ_m)
u_m ..... uncertainty of the measurement result
u_{CRM} ..... uncertainty of the certified value

The uncertainty of the measurement result (u_m) can be estimated with the help of the relative standard deviation of the repeatability (s_r) and d_2 (Section 2.3.1). Alternatively the uncertainty of the measurement results (u_m) can be estimated by dividing the standard deviation by the square root of the number of measurements carried out:

$$u_m = \frac{s_r}{\sqrt{n}} \qquad \text{Equation 8}$$

u_m ..... uncertainty of the measurement result
s_r ..... standard deviation of the repeatability
n ..... number of independent measurement results

The expanded uncertainty U_Δ, corresponding to a confidence level of approximately 95 %, is obtained by multiplication of u_Δ by a coverage factor $k = 2$.

$$U_\Delta = 2 * u_\Delta \qquad \text{Equation 9}$$

U_Δ ..... expanded uncertainty of difference between result and certified value
u_Δ ..... combined uncertainty of result and certified value

If $\Delta_m \leq U_\Delta$ then there is no significant difference between the measurement result and the certified value, meaning that the method does not have a bias. In case a bias was found the cause has to be investigated and eliminated. Approaches to calculate a bias can be found in GUM [2], but have to be considered carefully as a bias may be a constant or a factor.

The expanded uncertainties U_{CRM} of each certified value are given on the certificate of each individual reference material. The standard uncertainty, u_{CRM}, of the certified value is obtained by dividing the stated expanded uncertainty by the coverage factor given on the certificate.

Note: The Nordtest report [13] mentions a factor of 2.8 which should not be mixed-up with the coverage factor $k = 2$, leading to a confidence of about 95 %. Factor 2.8 can be used to

check if the MU estimated before is also applicable for measurements on a new sample. In case of inhomogeneity of the sample or the method being out of control, the before calculated MU might not be applicable any more. If the absolute difference between the two measurements is higher than 2.8 times the standard deviation, the MU calculation has to be reconsidered and /or the sample homogeneity questioned.
Furthermore, it should be noted that the CRM used for the bias control should not at the same time be used for calibration. In case this can not be avoided the analysis of a CRM with a low GM concentration calibrated with the diluted extracts of a CRM with a higher GM concentration should be considered.

2.3.3 Estimation of the uncertainty component associated with bias

Uncertainty associated with bias should, where possible, be estimated by the measurement of CRMs. The general approach is to measure the concentration of GM in a CRM in a number of analytical runs. An estimate of the uncertainty associated with bias is gained by combining the uncertainty associated with the mean measurement result with the uncertainty associated with the certified value of the concentration of GM in the CRM.

Note: In case of a bias the experimental set-up needs to be changed until no bias is found.

The relative standard uncertainty associated with the bias (u_{biasr}) is given by:

$$u_{biasr} = \sqrt{\frac{RSD_R{}^2}{n} + \left(\frac{u_{CRM}}{c_{CRM}} 100\right)^2} \qquad \text{Equation 10}$$

RSD_R..... relative within-laboratory reproducibility
n ..... number of measurements
u_{CRM} ..... standard uncertainty associated with the certified value of the CRM
c_{CRM} ..... certified value of the CRM

Note: U_{CRM} of each certified value is given on the certificate of each individual reference material. The standard uncertainty, u_{CRM}, of the certified value is obtained by dividing the stated expanded uncertainty by the coverage factor given on the certificate.

2.3.4 Calculation of the relative standard uncertainty

The relative standard uncertainty (*RSU*) is calculated by combining relative variation (RSD_R) and relative uncertainty associated with bias (u_{biasr}) using:

$$RSU = \sqrt{RSD_R^2 + u_{biasr}^2} \qquad \text{Equation 11}$$

RSD_R ..... within-laboratory reproducibility
u_{biasr} ..... standard uncertainty associated with bias$_r$

Note: The individual standard uncertainties need to have the format of a standard deviation in order to allow summing up. Independent uncertainties can be combined by taking the square root of the sum of the individual squares. *RSU* and u_{biasr} are not completely independent from each as all measurements are influenced by the RSD_R (Equation 10 and 11). The effect is considered to be negligible. Additionally it should be noted that in some cases either *RSU*, or u_0 can be zero.

The laboratory should generally explain how the MU has been calculated. Additionally the coverage factor applied and the corresponding uncertainty levels should be stated. A general explanatory note can be prepared to ease communication.

2.4 Evaluation of measurement uncertainty

Given of absolute standard uncertainty (u_0) and relative standard uncertainty (*RSU*) estimated from collaborative trial results (Section 2.2) or in-house quality control data (Section 2.3) then the standard uncertainty *u* associated with a measurement result *c* is given by:

$$u = \sqrt{u_0^2 + (c \times RSU)^2} \qquad \text{Equation 12}$$

u_o .....absolute standard uncertainty
c.....measurement result
RSU .....relative standard uncertainty

Note: It has to be stressed that Equation 12 is valid under the assumption that u_0 is constant and *RSU* proportional to the concentration *c*. This assumption should be checked using in-house validation data. A situation can occur, that u_0 is so small that it can be neglected (see example in Annex III).

Regulations (EC) No 1829/2003 and (EC) No 1930/2003 set a labelling threshold for the total authorised GMO presence on an ingredient basis. As such, the GMO contents for various events of one ingredient (e.g. MON 810 maize and 1507 maize) must be added together and the uncertainties associated with each individual GMO measurement combined. The MU of various methods can be combined by adding the squares and taking the square root of the sum:

$$u_c = \sqrt{\sum_{i=1,n} u_{meth,i}^2} \qquad \text{Equation 13}$$

u_c .....combined standard uncertainty associated with the measurement result for one ingredient
n .....number of method applied
$u_{meth,i}$ .....absolute standard uncertainty of individual method

The expanded uncertainty *U* (giving a confidence level of approximately 95 %) is given by:

$$U = 2 \times u_c \qquad \text{Equation 14}$$

u_c .....combined standard uncertainty associated with the measurement result for one ingredient

2.5 Calculation of the critical level

The critical level (LC) is the lowest measurement result that demonstrates with sufficient confidence that the analyte is present (Figure 3). LC is given by

$$LC = 2 \times u_0$$ Equation 15

u_o .....absolute standard uncertainty

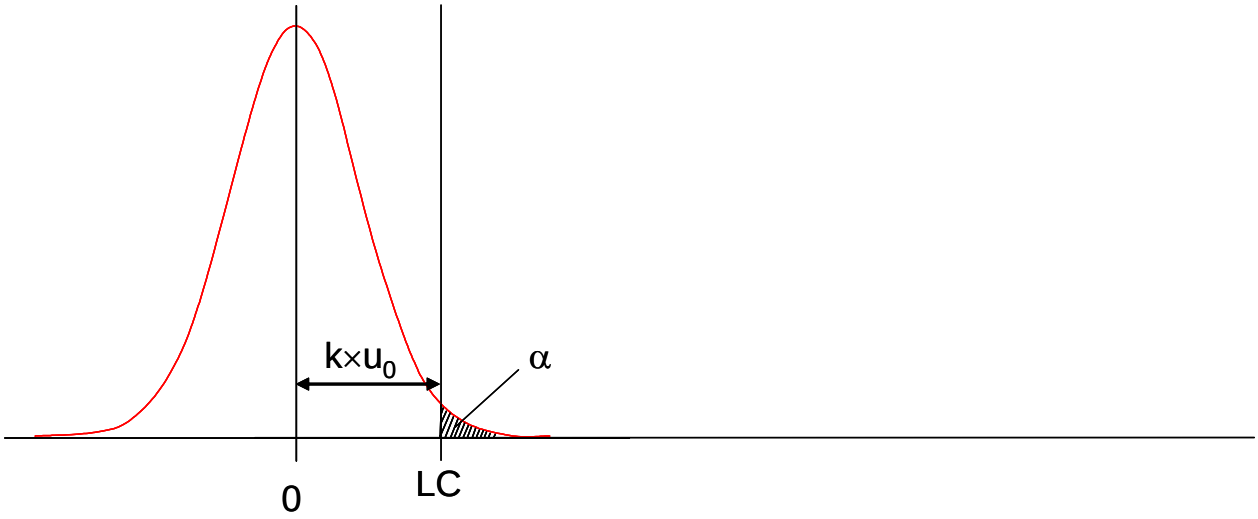


Figure 3: The LC is the lowest measurement result that demonstrates with sufficient confidence that the analyte is present (concentration > 0). If a value of *k* = 2 is used to estimate LC then the probability (α) of a sample containing none of the analyte giving a result above LC is 2.5 %.

2.6 Calculation of the limit of detection and quantification

Figure 4 illustrates the connection between LC and LOD. The LC is a statistically calculated number. If the result of the measurement is less than LC, then we can explain (with 95 % confidence) that the current sample does not contain any analyte. We use a *t*-statistic to decide if the expected value of GM concentration is 0 or not. If our measurement result is smaller than LC then we can report with 95 % confidence, that the sample does not contain any GM. If the measurement result is above LC, we are sure that GM is present. It should be noted that the probability to take a result for a blank is 50 % for measurement results below the LC.
While LC refers to the measurement result, the LOD and LOQ refer to the measured GMO concentration. Above the LOD, the probability to wrongly take a blank for a result is the same as for the LC while the probability to incorrectly interpret a signal for the presence of a GM is 5 %. It will be sure to detect a GM if the measurement result is above the LOD. The LOQ is the measured GM concentration at which the measurement uncertainty is below a certain (arbitrarily chosen; usually 30 %) value.

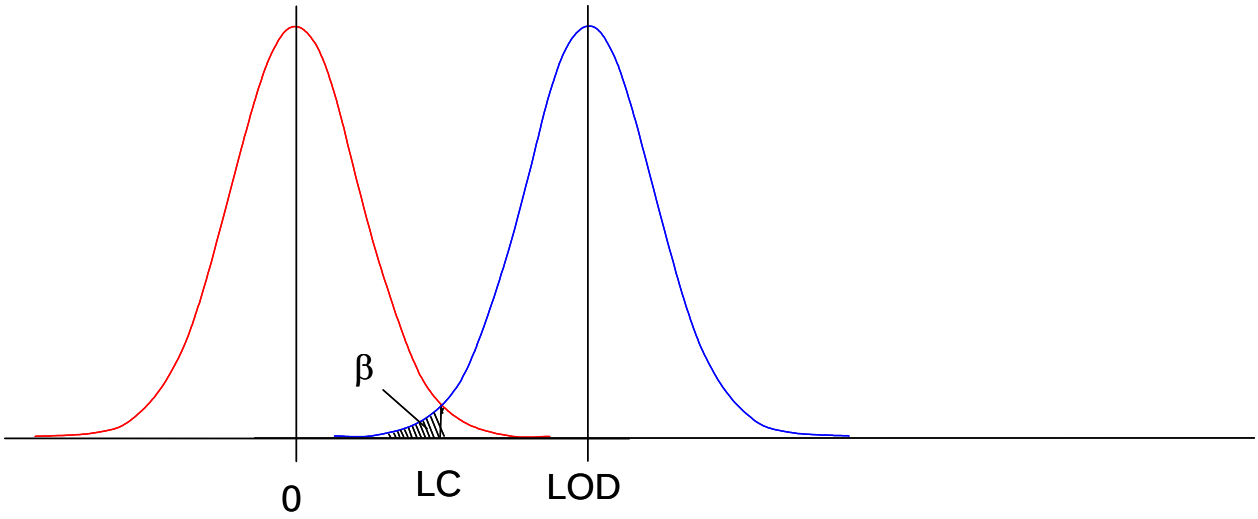


Figure 4: The LOD is the lowest true concentration that we can be sufficiently confident of detecting. This means it is the lowest true concentration that is unlikely to give a result below the critical level. If it is estimated as the lowest concentration where the lower limit of the expanded (k = 2) uncertainty is greater then the LC then the probability (β) of not detecting the analyte present at a concentration equal to the LOD is 2.5 %

The LOD is given by:

$$LOD = \frac{4u_0}{1-4RSU^2} \qquad \text{Equation 16}$$

u_o .....absolute standard uncertainty
RSU .....relative standard uncertainty

The LOQ is given by:

$$LOQ = \sqrt{\frac{u_0^2}{RSU_{MAX}^2 - RSU^2}} \qquad \text{Equation 17}$$

u_o .....absolute standard uncertainty
RSU .....relative standard uncertainty
RSU_{MAX} .....largest acceptable relative standard uncertainty

Note: Where the value of LOQ calculated using Equation 17 is less than the value of LOD then LOQ = LOD.
For the calculation of u_o the user needs to investigate whether the lowest concentration analysed is close to the LOQ of the method (Section 2.2).

Where RSU_{MAX} is the largest acceptable relative standard uncertainty that can be associated with results. By collaborative trial we can consider it as the greatest acceptable gradient of the regression line.

Note: LOD and LOQ are frequently estimated as multiples of u_0 with the LOD equal to 4-times u_0 and the LOQ 9-times u_0. This approach would require a constant u_0, as this is not given for values measured below the LOQ, the approach given here was favoured. However, estimating LOD and LOQ as multiples of u_0 could be still used, when the necessary precautions are taken.

2.7 Reporting results

Measurement results should be reported as follows:

Where *c* > LOQ

'$Concentration = (c \pm U)$
The uncertainty reported with this result is an expanded uncertainty calculated from a standard uncertainty using a coverage factor of 2. It is equivalent to a confidence level of approximately 95 %.'

Where LC < *c* < LOQ

Report '$Concentration = (c \pm U)$'unless it is not possible to estimate concentrations below LOQ (e.g. where LOQ is also the lowest point on the calibration curve).
If this is the case report the result as '$Concentration \le (\mathrm{LOQ} + \mathrm{U}_{\mathrm{LOQ}})$' where U_{LOQ} is the expanded uncertainty at a concentration equal to the LOQ.

Where *c* < LC

'$Concentration < \mathrm{LC}$
The target GM has not been detected in this sample.'

2.8 Estimation of the uncertainty associated with the mean

Where the *RSU* associated with an individual measurement result is estimated from the results of a collaborative trial ($RSU = RSD_R$) then the *RSU* associated with the mean of *n* independent replicates is given by:

$$RSU_n = \sqrt{RSD_R^2 - \frac{RSD_{rep}^2 (n-1)}{n}} \quad \text{Equation 18}$$

RSU_n .....RSU associated with the mean result
RSD_R .....relative standard uncertainty
RSD_{rep}.....relative standard deviation describing between-replicate variation
n ..... number of independent measurements

Where RSU_n is the relative standard uncertainty associated with the mean result and RSD_{rep} is the relative standard deviation describing between-replicate variation. The value of RSD_{rep} can be estimated using the method described in Section 2.3.1.

2.9 How to use measurement uncertainty

The MU report of DG SANCO [1] states that Enforcement Authorities shall use the measurement uncertainty associated with an analytical result when deciding whether an analytical result falls within the specification for food and feed control purposes. The way that MU is to be used by Enforcement Authorities must be taken into account when analytical specifications are discussed. In practice, the analyst will determine the analytical result and estimate the MU at that level. The value obtained by subtracting the uncertainty from the reported concentration, is used to assess compliance. Only if that value is greater than the maximum concentration stipulated in legislation, it is sure 'beyond reasonable doubt' that the sample concentration of the analyte is greater than that prescribed by legislation.

The estimated MU must be reported together with the measurement result. The uncertainty is of special importance, when the range of the expanded uncertainty encompasses the legal limit [11].

3 Further considerations

The authors consider that the two approaches given are efficient ones. However, other approaches to estimate the MU may be used. Furthermore, the authors wish to communicate further considerations which should be made when estimating the uncertainty linked to GMO quantification measurements by real-time PCR. In general these issues are currently recognised and discussed and their implications are evaluated at present.

Regulation (EC) No 1829/2003 and (EC) No 1930/2003 set a labelling threshold for the total authorised GMO presence on an ingredient basis. The MU and LODs associated with a sample are influenced by the level to which the ingredient itself is present. In compound food and feed the ingredient under investigation resembles less than 100 %. Although the GMO concentration of interest from a legislative perspective is the same, the number of DNA targets can be much lower in compound stuff, which can lead to quantifications close to the LOD.

Recent years have witnessed major advances with respect to technology and methods used to identify ingredients derived from GM materials, such as real-time PCR platforms and microarrays. However, this rapid advance in technological expertise has not been mirrored by a concurrent progression in the bio-analytical measurement community. Until now data handling of data obtained during GM detection has not be standardised [18].

The estimation of MU in any analytical approach attempts to identify all components that add significant variability to the end result. As such, MU estimation must take into account the whole analytical process from the initial generation of the result through to its final reporting. Whilst most MU estimation is focused on the practical experiments associated with the analytical process, little work has been done to explore the analysis of the data associated with such experiments. Example approaches to data handling that can contribute to MU are shown in Annex IV.

Statistical consideration of the amplification process used in quantitative PCR determinations suggests that the variation displayed by measurement results are described by a mixture of normal, binomial, and lognormal distributions, dominated by the latter two. A recent trial [19] has shown that GMO proficiency test results consistently follow a positively skewed distribution. Log-transformation prior to calculating *z*-scores is effective in establishing near-symmetric distributions that are sufficiently close to normal to justify interpretation on the basis of the normal distribution. It is therefore recommended that data from real-time PCR analyses should be log-transformed before calculation of the MU.

The estimation of the uncertainty associated with qualitative analysis of unauthorised GMOs is more difficult to determine than that associated with quantitative analysis of authorised GMOs because of the lack of validated methods, calibrants and CRMs. Generally acceptable

[18] Burns M, Valdivia H, Harris N (2004): Analysis and interpretation of data from real-time PCR trace detection methods using quantitation of GM soya as a model system, Analytical and Bioanalytical Chemistry, 378(6), 1616-1623

[19] Thompson M, Ellison SLR, Owen L, Mathieson K, Powell J, Key P, Wood R, Damant A (2006): Scoring in Genetically Modified Organisms Proficiency Tests Based on Log-Transformed Results, JAOAC, 89(1), 232-239

guidance on this topic is still under development. However, suggested guidance and an example of the estimation of LC and LOD for qualitative analysis is given in Annex IV.

Acknowledgements

The authors would like to thank Patricia Bonner, Andrew Damant, Emile Laurensse, Roberta Onori and Boldiszar Vadja from the ENGL, as well as Anja Bråthen Kristoffersen from the National Veterinary Institute (Olso, NO) for their valuable comments. At the time the authors would like to thank Andrej Blejec from National Institute of Biology (Ljubljana, SI), Hendrik Emons and Thomas Linsinger from the IRMM (Geel, BE) for their important contributions to this document.

ANNEX I: Definitions applicable to GMO analysis

Measurement Uncertainty (MU)
Parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand [20].

Notes:
1. The parameter may be, for example, a standard deviation (or a given multiple of it), or the half-width of an interval having a stated level of confidence.
2. Uncertainty of measurement comprises, in general, many components. Some of these components may be evaluated from the statistical distribution of results of a series of measurements and can be characterised by experimental standard deviations. The other components, which can also be characterised by standard deviations, are evaluated from assumed probability distributions based on experience or other information.
3. It is understood that the result of a measurement is the best estimate of the value of a measurand, and that all components of uncertainty, including those arising from systematic effects, such as components associated with corrections and reference standards, contribute to the dispersion.

Accuracy
The closeness of agreement between a test result and the accepted reference value (adopted from [21]).

Applicability
The description of analytes, matrices, and concentrations to which a method can be applied. (modified from [22]).

Bias
Difference between mean measured value from a large series of test results and an accepted reference value (a certified or nominal value). The measure of trueness is normally expressed in term of bias [12].

Critical level (LC)
The lowest measurement result that demonstrates with confidence that the analyte is present [modified from 23].

Dynamic range - Range of quantification
The range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Expanded measurement uncertainty
The expanded uncertainty (U) allows to calculate an interval within which the value if the measurand is believed to lie with a higher level of confidence. U is obtained by multiplying the combined standard uncertainties by a coverage factor k. The choice of the factor k is based

[20] ISO (1993): International vocabulary of basic and general terms in metrology, second Edition
[21] ISO/FDIS 3534-1 (2006): Statistics - Vocabulary and symbols - Part 1: General statistical terms and terms used in probability
[22] Codex CX/MAS 02/4 (2002): Proposed draft guidelines for evaluating acceptable methods of analysis
[23] IUPAC recommendation (1995): Nomenclature in evaluation of analytical methods including detection and quantification capabilities, Pure & Appl. Chem., 67(10), 1699-1723

on the level of confidence desired. For an approximate level of confidence of 95 %, k is always 2 if the degree of freedom is > 2 [adopted from 8].

Limit of Detection (LOD)
Limit of detection is the lowest concentration or content of the analytes that can be detected reliably, but not necessarily quantified (slightly modified from [24]). LOD is generally expressed as the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time ($\leq$ 5% false negative results).

Limit of Quantification (LOQ)
The limit of quantification of an analytical procedure is the lowest amount or concentration of analyte in a sample, which can be quantitatively determined with an acceptable level of precision and accuracy (modified from [24]).

Practicability
The ease of operations, in terms of sample throughput and costs, to achieve the required performance criteria and thereby meet the specified purpose (modified from [24]). Generally, the method should be practical for its intended purposes.

Repeatability standard deviation (RSD_r)
The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time (adopted from [21]).

Reproducibility standard deviation (RSD_R)
The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment [21].

Recovery
Proportion of the amount of analyte, present in or added to the analytical portion of the test material, which is extracted and presented for measurement.

Robustness
The robustness of an analytical method is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage (adopted from [25]).

Sensitivity
The sensitivity of a method is a measure of the magnitude of the response caused by a certain amount of analyte.
The method should be sensitive enough in order to be able to detect/quantify with respect to the thresholds as provided in the relevant legislation.

---

[24] ISO/FDIS 24276 (2005): International Standard, Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – General requirements and definitions
[25] EMEA (1995): Validation of analytical methods: definitions and methology, CPMP/ICH/381/95, http://www.emea.eu.int/pdfs/human/ich/038195en.pdf

Since sensitivity is method- and purpose-dependent it should be specified in the protocol. A reasonable goal for sensitivity is that required to meet levels specified in contracts, with a reasonable certainty that the level does not exceed the required limit.
Sensitivity as a term is used in two different ways - LOD and the slope of a curve. The use of the LOD is the preferred term to use as a measure of the ability of a method to detect a small amount of analyte.

Specificity
Property of a method to respond exclusively to the characteristic or analyte of interest.

Standard uncertainty
Uncertainty of the result of measurement expressed as a standard deviation [7].

Trueness
The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value (adopted from [21]).

ANNEX II: Example for an international collaborative trial to determine accuracy of a real-time PCR procedure

This Annex gives a detailed description of the concept and execution of an international collaborative trial in order to validate a real time PCR based method to quantify Roundup Ready® soya (RRS).

The collaborative trial was carried out in the year 2000. In total 31 laboratories took part in the trial, either using the ABI Prism® 7700 SDS (14 participants), ABI Prism® 5700 SDS (5 participants) or the LightCycler® system (12 participants).

In total five unknown samples containing between 0.1% and 5 % (m/m) of CRM IRMM-410R [26] and one sample consisting of texturized vegetable protein (TVP) containing 2 % (m/m) of the soya bean line GTS 40-3-2 were used in this trial.

The trial encompassed the whole procedure as needed for analysing a test sample, including the DNA extraction carried out according to a pre-scribed protocol using the classical CTAB solution and the PCR step. For each sample two DNA extractions have been analysed in parallel. Each test sample was analysed in 3 independent replicates. The mean value together with the standard deviation was reported.

For the relative quantification, separate standard curves were established for the reference target sequence and for the GMO target sequence. Therefore, DNA from 5 % (m/m) CRM IRMM-410R was extracted and used as a calibrant for both standard curves. At each of the four calibration points, duplicate (ABI PRISM® 7700 SDS and GeneAmp®5700) or single determinations (LightCycler System) were performed using a series of 1:4 dilution intervals.
Finally, the copy number measured for the unknown samples were obtained by interpolation from individual standard curves. Subsequently the percentages were calculated by dividing the copy number of the RRS copy number by the reference target sequence copy number and by multiplication with 100.

For primer/probe systems and PCR conditions, respectively, see details in ISO 21570 [16].

Data on specificity, linearity, LOD and LOQ have been determined prior to the collaborative trial.

Due to the concept of the trial, material from the same source was used as unknown samples (0.1, 0.5, 1, 2 and 5 % (m/m) CRM IRMM-410R) and as calibrant (5 % (m/m) CRM IRMM-410R). Therefore the set-up of the study is not well suited for the evaluation of the trueness of the applied real-time PCR method. However, the results could be used for assessing the precision of the applied method, whereby the described issues reflect a worst-case scenario resulting in an underestimation of the precision of the applied real-time PCR method.

---

[26] Trapmann S, Le Guern L, Kramer GN, Schimmel H, Pauwels J, Anklam E, Van den Eede G, Brodmann P (2000): The Certification of a new set of Reference Materials of Soya Powder with different Mass Fractions of Roundup Ready™ Soya, EC certification report EUR 19573 EN, ISBN 92-828-9639-0

As an example of the statistical evaluation of the results, Table 2 summarises the reported individual participant results using either the ABI Prism® 7700 SDS or ABI Prism® 5700 SDS. For checking significant outliers a Cochran and Grubs test was applied to the results reported by the participants. Both independent results for each sample were averaged and tested. After elimination of identified outliers for the 0.5, 1 and 5 % samples the mean quantities and standard deviation were calculated.

Table 2 Validation data for ABI PRISM ™ 7700 SDS and GeneAmp® 5700 SDS

Sample	Sample 1 0.1 (m/m) %	Sample 2 0.5 (m/m) %	Sample 3 1 (m/m) %	Sample 4 2 (m/m) %	Sample 5 5 (m/m) %	Sample 6 2 (m/m) % TVP
Number of laboratories	19	19	19	19	19	19
Number of outliers	0	2	1	0	1	0
Number of laboratories retained after eliminating outliers	19	17	18	19	18	19
Mean value [m/m %]	0.11	0.49	1.00	2.27	5.11	1.71
Bias [%]	9	-1	0	13	2	-15
Repeatability standard deviation s_r	0.04	0.12	0.21	0.25	0.53	0.48
Repeatability relative standard deviation RSD_r [m/m %]	33	24	21	11	10	28
Repeatability limit r ($r = 2.8 \times s_r$)	0.10	0.33	0.59	0.71	1.48	1.34
Reproducibility standard deviation s_R	0.05	0.3	0.28	0.71	1.38	0.55
Reproducibility relative standard deviation RSD_R [m/m %]	44	27	28	32	27	32
Reproducibility limit R ($R = 2.8 \times s_R$)	0.13	0.37	0.77	2.00	3.87	1.54

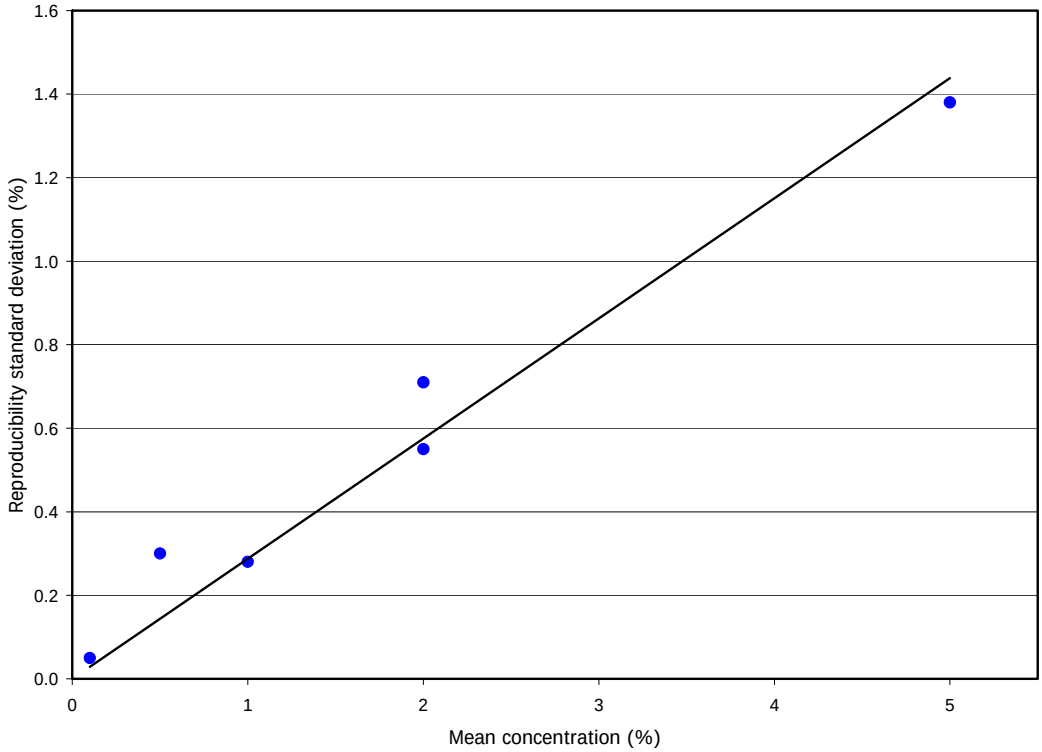


Figure 5: Mean concentration (*c*) plotted against s_R

In this case, the lowest analysed concentration is presumably lower than the LOQ. The value of u_0 is estimated from the value of s_R associated with the lowest concentration sample:

$u_0 = 0.05$

The value of RSU is estimated from the gradient of a linear regression line (with intercept set to zero)

$RSU = 0.29$

Hence the standard uncertainty associated with a measurement result c is given by (from Equation 14):

$$u = \sqrt{0.05^2 + (0.29 \times c)^2}$$

Hence, LC is equal to a measurement result of (from Equation 15) 0.10 % (m/m) GM

The LOD for the method is equal to a concentration of (from Equation 16) 0.30 % (m/m) GM.

If a relative standard uncertainty of less than 30 % is required for fit for purpose quantitative results then the limit of quantification is given by (from Equation 17):

LOQ = 0.6 % (m/m)

Given a measurement result 2.3 % (m/m) GM the expanded uncertainty is given by (from Equation 15):

$$U = 2 \times \sqrt{0.05^2 + (0.29 \times 2.3)^2} = 1.3 \text{ \% (m/m)}$$

Hence, the result should be reported as:

'(2.3 ± 1.3) % (m/m) GM

The uncertainty associated with the result is an expanded uncertainty calculated from a standard uncertainty using a coverage factor of 2. It is equivalent to a confidence level of approximately 95 %. The result minus the uncertainty is larger than 0.9 %.'

ANNEX III: Example for the evaluation of the measurement uncertainty based on single laboratory results

Evaluation of within-laboratory reproducibility

36 routine samples containing different concentration levels of RRS are analysed twice independently (Table 3) and are used to calculate the reproducibility standard deviation and relative standard deviation.

Table 3: Measurement results obtained on routine samples (n = 2) and calculation of the relative difference

Analysis number	GM concentration $c_{i,1}$ [m/m %]	GM concentration $c_{i,2}$ [m/m %]	mean $\bar{c}_i$ (Equation 1)	difference d_i (Equation 2)	relative difference rad_i [%] (Equation 3)
32	0.104	0.101	0.102	0.003	2.9
12	0.155	0.147	0.151	0.008	5.3
30	0.142	0.170	0.156	0.028	17.9
26	0.177	0.174	0.176	0.003	1.7
29	0.220	0.320	0.270	0.100	37.0
34	0.295	0.254	0.274	0.041	14.9
17	0.328	0.262	0.295	0.066	22.4
6	0.280	0.340	0.310	0.060	19.4
35	0.303	0.331	0.317	0.028	8.8
15	0.347	0.414	0.381	0.067	17.6
22	0.360	0.485	0.423	0.125	29.6
4	0.400	0.600	0.500	0.200	40.0
16	0.691	0.614	0.652	0.077	11.8
20	0.698	0.700	0.699	0.002	0.3
10	0.641	0.823	0.732	0.182	24.8
23	0.998	0.931	0.965	0.067	6.9
8	1.352	1.349	1.351	0.003	0.2
21	1.493	1.671	1.582	0.178	11.3
14	1.948	1.250	1.599	0.698	43.7
9	2.086	1.733	1.909	0.353	18.5
18	2.017	1.879	1.948	0.138	7.1
33	1.750	2.202	1.976	0.452	22.9
13	2.331	2.466	2.398	0.135	5.6
36	2.800	2.500	2.650	0.300	11.3
1	4.300	3.900	4.100	0.400	9.8
2	4.900	4.800	4.850	0.100	2.1
28	5.600	5.700	5.650	0.100	1.8
25	15.270	15.240	15.255	0.030	0.2
11	23.224	20.197	21.710	3.027	13.9
5	24.000	23.000	23.500	1.000	4.3
3	36.000	41.000	38.500	5.000	13.0
27	45.000	46.000	45.500	1.000	2.2
19	53.985	56.187	55.086	2.202	4.0
24	50.091	60.808	55.449	10.717	19.3
7	71.000	82.000	76.500	11.000	14.4
31	94.000	91.000	92.500	3.000	3.2

Here s_R is estimated from the six results with the lowest mean concentration and RSD_R is estimated from the remaining 30 results.

Hence,

$\bar{d} = 0.0304$

$$s_R = \frac{0.0304}{1.13} = 0.0269 \text{ \% GM}$$

$\overline{rad} = 13.1$ %

$$RSD_R = \frac{13.1}{1.13} = 11.5 \text{ \%}$$

Method and Laboratory bias control

For the bias control a CRM with a certified RRS concentration of (10.0 ± 1.6) g/kg was used (ERM-BF410d [27]). The certificate states that a coverage factor of $k = 2$ was applied. u_{CRM} is therefore 0.8 g/kg.

Six independent measurements ($n = 6$) were carried out (Table 4).

Table 4: Measurement results obtained on a CRM

Analysis number	GM concentration c [g/kg]	Mean GM concentration c_m [g/kg]	Standard deviation s [g/kg]
1	11.0		
2	10.9		
3	12.1	**11.1**	**0.5**
4	11.2		
5	10.7		
6	10.9		

The standard deviation (s) is divided by the square root of the number of measurements (n), as the average of the results is compared with the certified value. u_m is therefore estimated as 0.5/√6 g/kg = 0.2 g/kg.

$\Delta_m = |c_m - c_{CRM}| = |11.1 - 10.0|$ *g/kg* = 1.1 g/kg

$u_\Delta = \sqrt{u_m^2 + u_{CRM}^2} = \sqrt{0.2^2 + 0.8^2}$ *g/kg* = 0.82 g/kg

[27] Trapmann S, Catalani P, Conneely P, Corbisier P, Gancberg D, Hannes E, Le Guern L, Kramer GN, Prokisch J, Robouch P, Schimmel H, Zeleny R, Pauwels J, Van den Eede G, Weighardt F, Mazzara M, Anklam E.(2002): The Certification of Reference Materials of Dry-Mixed Soya Powder with different Mass Fractions of Roundup Ready™ Soya, EC certification report EUR 20273 EN, ISBN 92-894-3725-1

The expanded uncertainty U_Δ is 2 u_Δ = 1.64 g/kg. This is larger than the difference Δ_m between the certified and the measured value. The measured mean value is therefore not significantly different from the certified value and it can be concluded that the method has no bias.

Uncertainty associated to the bias

Any bias that may be associated with results is less than the uncertainty associated with bias. The standard uncertainty associated with this (u_{biasr}) is calculated as (Equation 10):

$$u_{biasr} = \sqrt{\frac{11.5^2}{6} + \left(\frac{0.8 * 100}{10.0}\right)^2} = 9.3\ \%$$

For
n 6
RSD_R .... 11.5 %
u_{CRM} ..... 0.8 g/kg (U (k=2) = 1.6 g/kg)
c_{CRM} ..... 10.0 g/kg

Calculation of the relative standard uncertainty

The relative standard uncertainty (*RSU*) is calculated by combining RSD_R with the u_{biasr} (Equation 11):

$$RSU = \sqrt{11.5\%^2 + 9.3\%^2} = 29.6\%$$

Evaluation of the measurement uncertainty

Given that for the sample under investigation a RRS concentration of 15 g/kg has been measured, the standard measurement uncertainty is calculated according to Equation 12. As outlined in Section 2.2, s_R associated with the lowest concentration can be used as an estimate of u_0:

$$u = \sqrt{0.0269\%^2 + (15\,g/kg \times 29.6\%)^2} = 2.22\ g/kg$$

The value for u_0 indicates already that it in this specific case it can be neglected. Hence, a direct calculation as outlined in the Nordtest report [13], following Equation 11 would be also possible:

$$RSU = \sqrt{9.3\%^2 + 11.5\%^2} = 14.79\ \%$$

$$u = 14.79\% * 15\,g/kg * \frac{1}{100} = 2.22\ g/kg$$

The expanded uncertainty is calculated according to Equation 14 using a coverage factor of 2, which gives a level of confidence of approximately 95%:

$$U = 2 \times 2.22\,g/kg = 4.44\ g/kg$$

Final step and reporting

Given that for the sample under investigation a RRS concentration of 15 g/kg has been measured, the analytical result is reported as:

'Roundup Ready® soybean = (15 ± 5) g/kg

The expanded MU of relative 30 % is estimated from two independent analyses of real samples. A coverage factor of 2 was used, corresponding to a confidence level of approximately 95 %. The method applied proved to have no bias.'

ANNEX IV: Example of data handling operations potentially leading to uncertainty

This list is by no means exhaustive, but it provides examples of which areas of data handling would benefit from increased standardisation, in order to reduce MU associated with a reported result. This list should raise awareness regarding the number of data handling aspects that can potentially give rise to MU. Additionally, although a number of current projects (e.g. the European sixth framework programme: Co-Extra [28]) are attempting to address the area of standardised data handling associated with GM results, there is little published literature relating to standardised guidelines on the subject [18, 29]. Thus, the following areas of data handling have been emphasised as potential factors that can account for MU in a reported result, but additional work needs to be conducted in the GM and real-time PCR fields in order to provide a standardised way to approach data handling.

- A calibration curve is often constructed by measuring an instrument response according to a range of reference standards of known analyte concentration. The concentration of the analyte in the sample unknown is then evaluated based on this calibration curve. The construction of the calibration curve can affect the reliability of the result, and it needs to be considered if the curve should be produced using average values, or values from individual independent replicates.
- The model used to construct the calibration curve is another important consideration. Typically, simple linear regression is used to produce the 'best fitting straight line' by minimising the sum of the squares of the deviations between the observed and expected values, given that a linear relationship exists. However, simple linear regression does not take into account the variability associated with each standard on the curve. Whilst in some experiments, linear regression is sufficient to give an acceptable response, it can bias results particularly when the variability of standards at the extreme ends of the calibration curve have different precision. This often occurs in routine GM analysis, and statistically, alternative models that take into account this heterogeneity of variance would be more suitable, for example weighted regression or censored regression.
- The majority of reported results associated with GM quantification are based on using singleplex reactions within the laboratory. Singleplex reactions occur where the endogenous target analyte and the transgenic target analyte are assessed in separate wells or reaction vessels. However, an alternative approach is to use duplex reactions, where both endogenous and transgenic target analytes are assessed in the same reaction vessel. These two approaches have different characteristics associated with them in terms of use of resources and costs, and competition between reactions. However, the different reaction kinetics of the two approaches also dictate that the data can be evaluated according to a variety of methods, and one method may not be the most appropriate way to handle both sets of data.
- The analyte concentration of sample unknowns are typically estimated based on the regression Equation associated with the calibration curve. Because of the nature of the

[28] Homepage of the European FP6 project Co-Extra – GM and non-GM supply chains: their co-existence and traceability: http://www.coextra.eu

[29] Burns MJ, Nixon GJ, Foy CA, Harris N (2005): Standardisation of data from real-time quantitative PCR methods - evaluation of outliers and comparison of calibration curves, BMC Biotechnology, 5:31 doi: 10.1186/1472-6750-5-31

PCR data set and the target analyte concentrations involved, this normally involves log transformation of data. A guidance document relating to how to evaluate sample unknowns would be beneficial, as taking individual independent replicates and then log transforming to give an average value, can give a very different result to averaging individual replicate values before log transformation. Additionally, the confidence level with which the final result is estimated will also differ according to what approach to transforming data has been adopted.

- To help ensure that reaction conditions are similar between calibration curves, the slope of calibration curves are often compared. This gradient of the calibration curve is indicative of the efficiency of the PCR reaction, and for quality purposes it is also important that the PCR efficiency of standards used to produce a calibration curve is similar to the PCR efficiency associated with sample unknowns. This comparison has typically been conducted using a subjective visual assessment, but a statistical approach based on analysis of co-variance has been published that facilitates a more objective evaluation. The production of guidelines on how to facilitate this could impact upon the assessment of uncertainty in the reported result.
- Individual PCR measurements in a data set that are thought to be outliers have the potential to introduce bias into a result. Standardised approaches to aid in successful identification of outlying values given the typical non-normal distribution of some values from real-time PCR, is only part of the issue associated with this problem. The second half of the problem involves recommending an approach to facilitate an objective decision on whether to include or exclude suspected outlying values, and this can impact greatly upon the final measurement. An objective approach using a combination of graphical and statistical techniques followed by identification and handling of outlying values using ISO guidelines, has been published [29].

The limited list of data handling approaches described above requires careful thought regarding the best statistical approaches to help interpret data. More work needs to be done at an international level in order to provide a standardised guidance document that would address all data handling approaches in this field. Production of such a standardised 'best practice guidelines' document will facilitate more accurate analysis of data arising from GM detection methods, and will contribute towards a greater understanding of the MU inherent within different data handling approaches. This will help provide a more meaningful and clearer interpretation of data arising from GM detection methods than is currently available.

ANNEX V: Data calculated on the bases of CRL collaborative trials

Table 5: LOD, LOQ, *U* and estimated enforcement level based on collaborative trial data

GM event	Matrix used for validation (DNA extraction method)	Measurement unit used during validation	LOD [%]	LOQ [%]	Expanded Uncertainty U (k = 2) at labelling threshold (0.9 %) [%]	Estimated enforcement level [%]
Ring trial Roundup Ready (from Annex II)						
RUR	flour (CTAB)	mass fraction GM	0.30	0.65	0.53	1.92
CRL ring trails <u>including</u> DNA extraction step						
NK603 maize	flour (CTAB)	mass fraction GM	0.15	*	0.66	3.33
GA 21 maize	flour (CTAB)	mass fraction GM	0.36	*	0.58	2.46
MON 863 maize	flour (CTAB)	mass fraction GM	0.004	0.004	0.34	1.46
CRL ring trials <u>without</u> DNA extraction step						
Bt 11 maize	DNA	copy number ratio	0.11	0.09	0.31	1.38
TC1507 maize	DNA	copy number ratio	0.10	0.10	0.42	1.67
T25 maize	DNA	copy number ratio	0.01	0.01	0.43	1.72
DAS 59122 maize	DNA	copy number ratio	0.25	0.22	0.25	1.21
RUR H7-1 sugar beet	DNA	copy number ratio	0.12	0.10	0.26	1.27
281-24-236 cotton	DNA	copy number ratio	0.14	0.13	0.31	1.35
3006-210-23 cotton	DNA	copy number ratio	0.15	0.15	0.40	1.62
LL62 rice	DNA	copy number ratio	0.13	0.11	0.25	1.24

* RSU is bigger than RSU_{max}. The method is not good enough to fulfil the set criteria. Either the criteria have to be set less strict or the methods have to be improved.

Furthermore it should be noted that reliable estimation of the enforcement levels requires the inclusion of the DNA extraction. Therefore estimated enforcement levels based on ring trials using extracted DNA may underestimate the uncertainty and hence the estimated enforcement level.

The data provided here can be used if the method used in the laboratory is exactly the same. Details related to the method validation can be found on the homepage of the CRL for GMO food and feed [17].

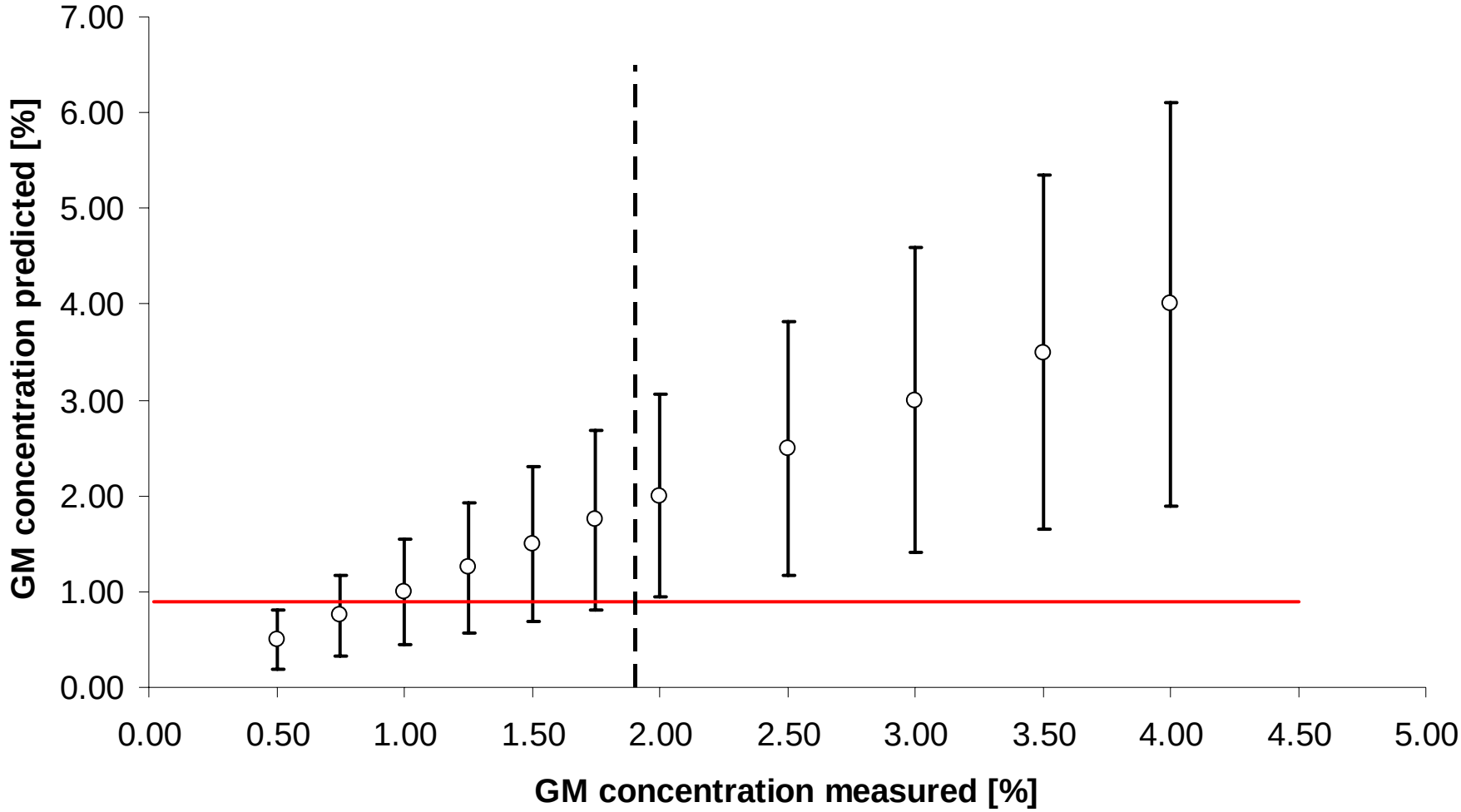


Figure 6: Enforcement level calculated for RRS collaborative trial given in Annex II, expressed in mass/mass %

Using the data obtained during the method validation of RRS by collaborative trial (Annex II), it can be concluded that samples for which a GM concentration above 1.8 % (dashed line) is measured (using the RRS method) contain more that than the legal threshold of 0.9 % GM (red line).

European Commission

EUR 22756 EN – DG Joint Research Centre, Institute for Reference Materials and Measurements –
Guidance Document on Measurement Uncertainty for GMO Testing Laboratories
Authors: S. Trapmann, M. Burns, H. Broll, R. Macarthur, R. Wood, J. Zel
Luxembourg: Office for Official Publications of the European Communities
2007 – 41 pp. – 21.0 x 29.7 cm
EUR - Scientific and Technical Research series; ISSN 1018-5593
ISBN 978-92-79-05566-9

Abstract

This technical report outlines the technical issues related to the estimation of measurement uncertainty (MU) involved in the GMO sector. In particular it gives guidance to GMO testing laboratories how to estimate the analytical variability of quantitative analytical results obtained by real-time PCR. This guidance document has been written on request of the European Network of GMO Laboratories (ENGL) as a follow-up of a workshop on Measurement Uncertainty in the GMO sector organised by the Institute for Reference Materials and Measurements (IRMM), Geel, Belgium and held on 05.07.2005.

It is recognised that in order to be able to judge if an analytical results exceeds a threshold; the MU must be estimated and reported together with the measurement result. Enforcement Authorities shall therefore estimate the MU associated with an analytical result and use it to decide whether an analytical result falls within the specification of food and feed control. The value obtained by subtracting the expanded uncertainty from the reported concentration is used to assess compliance. Only if this value is greater than the legal threshold, it is sure 'beyond reasonable doubt' that the sample concentration of the analyte is beyond what is permissible.

Two selected approaches for the estimation of MU are presented in detail; references to alternative approaches are given. The first approach presented in detail is using data from collaborative trial in combination with in-house quality control data for the estimation of MU. Prerequisites for the use of such collaborative trial data are outlined. In case no suitable collaborative trial data are available, an alternative approach using data from within-laboratory samples for the estimation of MU is presented.

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

The mission of the Joint Research Centre is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of European Union policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Community. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

15 LA-NA-22756-EN-C

ISBN 978-92-79-05566-9

Publications Office
Publications.eu.int